Author's response to reviews

Title: Water extract from processed Polygonum multiflorum modulate gut microbiota and glucose metabolism on insulin resistant rats

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Author's response to reviews:

Reviewer #1:

The authors have successfully addressed all the comments and questions raised by the reviewers and now the manuscript may be accepted for publication

Reviewer #2:

The authors have addressed to the concerns of the reviewers satisfactorily and made necessary revision in the revised draft. The revised draft is acceptable for publication

Reviewer #4:

PEER REVIEWER ASSESSMENTS:
OBJECTIVE - Full research articles: is there a clear objective that addresses a testable research question(s) (brief or other article types: is there a clear objective)?

Yes - there is a clear objective

DESIGN - Is the current approach (including controls and analysis protocols) appropriate for the objective?

Not sure - key details are missing from the manuscript

Responds: Thanks to the reviewer's precious advice. According to the opinion, We submitted some additional data to increase the details of our experiment.

EXECUTION - Are the experiments and analyses performed with technical rigor to allow confidence in the results?

No - there are major issues

Responds: Thanks to the reviewer's precious advice. According to the opinion, We submitted new evidence to prove the reliability of the results.

STATISTICS - Is the use of statistics in the manuscript appropriate?

Yes - appropriate statistical analyses have been used in the study

INTERPRETATION - Is the current interpretation/discussion of the results reasonable and not overstated?

No - there are major issues

Responds: Thanks to the reviewer's precious advice. According to the opinion, We submitted new evidence to prove the reliability of the results.

OVERALL MANUSCRIPT POTENTIAL - Is the current version of this work technically sound? If not, can revisions be made to make the work technically sound?

Maybe - with major revisions

Responds: Thanks to the reviewer's precious advice. According to the opinion, We adjusted the presentation of some experimental data to make the results more reasonable.
PEER REVIEWER COMMENTS:

GENERAL COMMENTS:

Minor issues

* What was the age of rats when experimentation began?(Page-5 paragraph-4 line-1)

Responds: Thanks to the reviewer's precious advice. At the beginning of the experiment, we used 5-6 weeks old Male Sprague Dawley rats(weighing of 180±20 g). we have already added this detail in the article.

* MET is not defined prior to first use (Page 6 line 113 and 118) but is presumably metformin. Please define for clarity.(Page-4 paragraph-2 line-2)

Responds: We agree with what the reviewer described. According to the opinion, the sentence has been adjusted, we re-describe the meaning of Metformin hydrochloride(MET) in the article.

* Since the one-way ANOVA reported is a multiple comparison test, was any post-hoc test such as Tukey used? Please specify.(Page-8 paragraph-3 line-3)

Responds: Thanks to the reviewer's precious advice. According to the opinion, We added statistical methods for data processing. when we used one-way ANOVA reported to identify significant differences between groups, two types post-hoc test was used(When the data variances is equal, LSD:1 method is used .When the data variance is not equal, Tamhanes T2 test is used).

* There are some minor stylistic issues throughout, but I have not listed them here as my opinion has specifically been sought to assess the methods and validity of the results.

Responds: Thanks to the reviewer's precious advice. Based on the comments, we re-described some sentences to make it as clear as possible.

Major Issues

* I am concerned about how the experiments on every 2nd week were planned. If blood had to be collected from the ocular vein every two weeks as shown in figure 1, why was a small sample from this procedure not used to measure fasting blood glucose as well? I don't understand why the authors chose to test FBG using blood the tail vein. This additional procedure adds unnecessary stress to the animals (both during restrains and blood collection). The only scenario I can think of where conducting both procedures (ocular and tail vein blood collection) becomes necessary is if the authors also intended to perform an oral glucose tolerance test (OGTT) which
typically uses tail vein blood samples at 0, 30, 60, 90 and 120 minutes. However, neither the method nor the data for OGTT have been presented.

If OGTT was attempted, this complicated the data. Ocular blood sampling every two weeks then would coincide with the OGTT timeline. In such a scenario, the animals will be extremely stressed and may negatively impact the results. Typically, such scenario can be avoided by having a larger number of animals per group (perhaps 12 or 15) and dividing them for blood collection and OGTT. Doing two blood collection procedures on the same animals so frequently in such a short span is not recommended. The negative impact of such a procedure (if any) may be observable in body weight change before and after the procedure. Was this record kept? I know from the experience in our laboratory that metabolic parameters, including insulin and glucagon concentrations and especially insulin, can be compromised in animals that are stressed due to frequent blood collection (both ocular and tail vein) although this may reflect in blood glucose concentrations only under extreme stress. So FBG concentrations can be more or less reliable, but I am concerned about insulin concentrations. I cannot say with certainty if gene expressions or microbiota data are affected by these procedures. (The issue with blood collection was also raised by the 2nd reviewer, but the response did not clarify these issues)

Responds: Thank you so much for your academic guidance and advice. We attached great importance to the protection of animal ethics and welfare.

Blood is taken every two weeks to observe the animal's modeling and to ensure the success of the model. We are sure that the modeling method is feasible from our previous experience and related literatures (see the below).

Certainly, we highly appreciate your suggestions on the frequency of blood collection to reduce the damage caused to animals. Therefore, in the future experimental design, we will reduce the frequency of blood collection and reduce the amount of blood samples.

When we take blood from the tail vein to measure fasting blood glucose, we use the blood glucose meter. Only the light tail of the rat can be used to obtain the fasting blood glucose level, and the damage to the rat is very small. In the subsequent experiment, we will also pay attention to these operations.

Here I supplemented the data of body weight change before and after the procedure. It can be seen from the figure that there is no significant effect on the body weight of rats before and after the procedure. Thanks to the reviewer's proposal, in the future experimental operation, we will design the experiment more rationally.

Thanks to the reviewers for their valuable suggestions. We also consulted the literature to understand the effects of frequent blood collection on insulin and other indicators in rats. Therefore, we will cautiously considered the frequency of blood taken when designing the experiment.
Fig.S1 Weight changes. Weekly weight changes in each group of rats from 0 week to 12 weeks.

For example 1: Singh S B, Selvamurthy W. Effect of intermittent chronic exposure to hypoxia on feeding behaviour of rats[J]. International Journal of Biometeorology, 1993, 37(4):200-2. (Blood samples were drawn once a week from the retro-orbital venous plexus for blood sugar analysis.)

For example 2: Shanhong Lu, Ting Zhang, Wen Gu, Xingxin Yang, Jianmei Lu, RonghuaZhao*, Jie Yu*, Volatile oil of Amomum villosum inhibits non-alcoholic fatty liver disease via the gut-liver axis, BioMed Research International, 2018. (Blood samples (about 1.5–2.0 mL) were collected from the retroorbital venous plexus of rats every 2 weeks and then centrifuged at 10,000 rpm for 15 min.)

For example 3: Pei Lin, Jianmei Lu, Yanfang Wang, Wen Gu, Jie Yu*, Ronghua Zhao*, Naturally Occurring Stilbenoid TSG Reverses Non-Alcoholic Fatty Liver Diseases via Gut-Liver Axis, PLoS ONE 10(10): e0140346, 2015. (Samples of blood were collected from the retroorbital venous plexus once every 6 days, two hours after administration of therapeutic agents in the morning.)

* Were the H and E staining done in different batches or different camera settings used? The dye uptake seems to vary considerably between panels A, B, E, and C&D. Such variation is not expected. Panel B (MOD group) also show signs of portal inflammation. However, this is not mentioned in the results. Also, this representative slide does not show increased vacuolated lipid droplets, as claimed by the authors. Lipid vacuoles are typical round. I can see more fatty deposits in Panel D (treatment group) thank in Panel B. (Page-9 paragraph-1 line-1)

Responds: Thank you so much for your academic guidance and advice. We attached great importance to this advice.

We added a description of group B portal inflammation in the article.

After consulting the relevant experts, we re-select the pathological slice picture under the 10X microscope, In order to observe the lipid droplets in a larger field of view (see below). In the figure, I marked the lipid vacuole with a red square. It can be seen that there are more lipid droplets in the model control group (B), and the number of lipid droplets is significantly reduced after administration.

In addition, the reviewer proposed the difference in the dye uptake. When we conducted this part of the experiment, we commissioned other specific department to help us with dye observation. Since the samples were not the same batch of dyeing, the dye uptake was different. In our future research, We will pay more attention to the parallelism of the samples.
Fig.2 Effect of PPM in liver tissue of insulin-resistant rats. The MOD group had obvious fatty degeneration in the liver cells, while the MET, PPM group was less severe. (A) normal control group, (B) model control group, (C) positive control group, and (D, E) group treated with 0.405 and 1.62 g·kg⁻¹ PPM.

* I must stress that these comments do not warrant that this paper is rejected. I urge the editor to seek clarifications on these issues and if satisfied this paper can be a useful addition to the existing literature on Polygonum multiflorum.

Responds: Thank you so much for your academic guidance and advice. Your comments was highly insightful and enabled us to greatly improve the quality of our manuscript. According to the opinion, The article has been modified, the modified parts of the article are marked in red font for easy viewing.